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ORIGINAL ARTICLE

Development of species identification tests targeting the 16S ribosomal RNA coding region in mitochondrial DNA

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Abstract This report describes the development of a species testing system based on the diversity of nucleotide sequences in mitochondrial DNA (mtDNA) among species. Five species, human, cow, pig, dog, and cat, were considered. The partial nucleotide sequences in 16S ribosomal RNA coding region were chosen as the target for discriminating the species. The sequence diversities of this approximately 400 bp long region ranged from 15.7 to 24.1% among the five species. Sequencing analysis of this target on 50 individuals of each species (53 for dogs) revealed that the nucleotide sequences were well preserved within species. Species-specific PCR for each species was also designed, and satisfactory results with regard to both sensitivity and specificity were obtained. A validation study with DNA extracted from bovine bone exposed to the environment revealed that the PCRs designed in this study worked correctly. From the results obtained, it was shown that this testing system could be a good tool for species identification. One successful case report is also demonstrated.

Keywords Species identification · 16S riobosomal RNA · Competitive PCR · Bone identification · Mitochondrial DNA

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Introduction

Species identification is an important issue in forensic sciences as a primary identification procedure in casework on biological specimens such as bloodstains, hair, and bone particles. Although this has conventionally been achieved by immunological and eletrophoretic tests targeting particular species-specific proteins, the progress of molecular biology introduced a new approach, which is based on nucleotide sequence diversities among species in particular regions of DNA [1–5, 7].

Due to advantages in detectability and specificity, this DNA-based method expanded its applications to a variety of purposes, such as in maintaining food authenticity by checking species content shown on labels of products [8–11], in avoiding the spread of bovine spongiform encephalopathy by assuring the lack of the bovine or ovine-derived materials in ruminant feedstuff [12–15], and in protecting endangered animals from illegal trade by identifying body parts (skin, fangs, meat, etc.) [16–18]. Besides these applications, the nucleotide regions chosen for species identification were also varied by researchers. Within vertebrates, a cytochrome *b* (cyt *b*) gene in the mitochondrial DNA (mtDNA) was commonly chosen [1–4, 7, 8, 12, 16, 17] as its suitability for species identification has been studied from multiple viewpoints including the nucleotide diversity among species [2] and the availability of nucleotide sequence data for references [1]. Many of the other regions studied are also located in the mtDNA. The coding regions for 12S and 16S ribosomal RNA (rRNA) [6, 10, 11, 15], ATPase subunits 8 and 6 [13, 14], and the noncoding D-loop region [3, 5, 9] have shown their potential to be the targets for the species test.

In this study, we assessed the usability of the partial nucleotide sequence of 16S rRNA coding region in mtDNA as a marker for discriminating several animal species commonly studied in forensic casework. Universal (species-independent) and species-specific PCRs were generated as testing tools. Validations on these PCRs were made from the aspects of sensitivity, specificity, and applicability to degraded DNA. Furthermore, a successful case report using these PCRs will also be demonstrated.

Materials and methods

Samples and DNA extraction

Human buccal swabs were collected from 50 Japanese individuals. Informed consent was obtained from each donor according to the guidelines of the ethical review board of the National Research Institute of Police Science, Japan. For animal DNA sources, residues of blood tests of cow, pigs, dogs, and cats were kindly provided by various animal hospitals and livestock hygiene service center in Japan. Sample numbers and breed of each animal are listed in Table S1. DNA was extracted from each specimen using a QIAamp DNA Micro Kit (Qiagen, Tokyo, Japan) according to the manufacturer's instruction. The DNA extracts were adjusted to 50 μl in volume with TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 7.5), and kept at −80°C until use.

Alignment of nucleotide sequences of mtDNA

Entire nucleotide sequence data of mtDNA for the five species were obtained from the GenBank [19]. The species and their accession numbers were as follows: humans (*Homo sapiens*): NC001807, cows (*Bos taurus*): V00654, pigs (*Sus scrofa*): AF034253, dogs (*Canis familiaris*): U96639, and cats (*Felis catus*): U20753. The sequence data were aligned using ClustalX software [20] version 1.83.1 under default analyzing parameters.

Designing of universal PCR primers

From the results of the alignments, two of the highly conserved regions consisting of identical nucleotide sequences in five species were chosen as the priming sites for the universal PCR, which were supposed to amplify the same target in wide ranges of animal species and allowed species identification by sequencing analysis. A partial region of a 16S rRNA coding region, of which the length ranged from 402 to 409 bp (due to species), was chosen in this study.

Universal PCR and sequencing

A universal PCR was carried out using a GeneAmp PCR system 9700 (Applied Biosystems, Tokyo, Japan) at 95°C for 9 min followed by 33 cycles of 95°C for 45 s, 58°C for 30 s, and 72°C for 2 min. Of the DNA extract, 1 μl was amplified in 25 μl of the PCR reaction containing 1× PCR GOLD buffer, 200 μM dNTPs, 1 U of AmpliTaq GOLD DNA polymerase, 2.5 mM $MgCl_2$ (Applied Biosystems), and 0.5 μM of each primer. The nucleotide sequences of the primers used are shown in Table S2. To assess the success of the amplification, 3 μl of each product was separated by electrophoresis in 2.0% SeaKem ME agarose gel (FMC BioProducts, Rochland, ME, USA) in 1× TAE buffer (40 mM Tris–acetate, 1 mM EDTA; Life Technology, Grand Island, NY, USA) for 20 min at 100 V. The gel was stained with ethidium bromide, and the DNA bands were visualized with a Bio Image GelPrint 2000i (Genomic Solution, Ann Arbor, MI, USA). The PCR products were then purified using Performa DTR Gel Filtration Cartridges (Edge Biosystems, Gaithersburg, MD, USA), and nucleotide sequences were determined with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instruction. The primers for sequencing were identical to those for the PCR. Both the heavy and the light chains were analyzed independently, and their nucleotide complementarities were confirmed.

Designing of species-specific PCR

From the results of the sequencing of multiple individuals in the target regions of the universal PCR, the most frequently observed nucleotide sequences were chosen in each species. These were identical to those obtained from the GenBank. The alignment was performed on these five sequences, and the possible regions for selective priming sites were chosen, considering the PCR conditions predicted by Oligo 4.06 primer analysis software (National Bioscience, Plymouth, MN, USA), as well as the intraspecies sequence consistency. The selective primers were designed for both the forward and reverse directions in every species-specific PCR. The nucleotide sequences are also listed in Table S2. Chemical compositions and thermal condition of species-specific PCR were consistent with the universal PCR, except that the annealing temperature was dropped to 54°C in the dog-specific PCR.

Competitive PCR

To assess the sensitivity of the species-specific PCR designed in this study, a competitive PCR method was applied for preparing the template DNA containing defined amounts of amplifiable mitochondrial DNA. An internal

standard with the same priming sites as the original PCR but 20 bases shorter in product size was generated for the universal PCR. This preparation was established according to the methods previously reported [21, 22]. Briefly, the DNA from cow was firstly amplified with a modified primer set consisting of one original primer and an internal primer tagged with a partial sequence of the original primer. The nucleotide sequence of this tagged internal primer used as the alternative to the reverse primer in the universal PCR was 5′-GTAACTTGTATCAATGAGCGATAGAGTGA-3′. Subsequently, a 1/50 dilution of the product was secondarily amplified with the original primer set. The internal standard generated was purified with a Centricon YM-100 device (Millipore, Billerica, MA, USA) and quantified using a Du-640 UV spectrophotometer (Beckman, Tokyo, Japan). The obtained value was converted into a copy number from its molecular weight, and the internal standard was subsequently adjusted to 1,000 copies/μl. The competitive PCR was carried out in the mixture of the 1,000 copies of the internal standard and the serially diluted DNA of five animal species. The products were separated using an Agilent 2100 bioanalyzer with a DNA 500 LabChip Kit (Agilent Technologies, Palo Alto, CA, USA).

DNA extraction from degraded bone samples

To validate the PCRs designed in this study on degraded materials, DNA was extracted from bovine metacarpal bones which had been left in the open air (placed in a cage in an open field), in water (immersed in an aquarium equipped with a water circulating system), and in soil (buried in garden soil at a depth of 30 cm) for 3 years. Compact bones were taken from the exposed samples, and the surface was sanded by grindstone and pulverized using a Multi-Beads Shocker (Yasui Kiki, Osaka, Japan). The bone powder was weighed, and 0.5 g of each was decalcified with 0.5 M EDTA at 56°C overnight. After removing excess EDTA by washing the powder twice with sterilized water and once with TNE buffer (10 mM Tris–HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0), powdered specimens were digested with 3 ml of TNE buffer containing 0.5% sodium dodecyl sulfate (SDS, Nippon Gene, Tokyo, Japan) and 0.5 mg/ml of proteinase K (Wako, Osaka, Japan). The DNA was isolated by phenol-chloroform extraction method, and the extract was concentrated using a Centricon YM-100 device. The final extract was adjusted to 100 μl with TE buffer, kept at −80°C until use, and 1 μl of each extract was added to the reactions as template.

Results and discussion

Alignment of the entire mtDNA sequence in five species

As a result of the alignment on the entire mtDNA sequence in five species, a total of 16 regions were found as highly conserved regions, which consisted of more than 20 continuously shared nucleotides. The nucleotide positions, length, and name of the gene are summarized in Table S3. Most of them were located in ribosomal RNA coding regions, three were in 12S rRNA region, ten were in 16S, and the remaining three were in transfer RNA coding regions. It should be noted that no such highly conserved region was found in the Cyt *b* region, which is commonly used as a means for species identification [1–4, 7, 8, 12, 16, 17]. This finding no doubt supports the high potential of Cyt *b* region as a tool for species identification. However, it also suggests a difficulty in designing a universal primer that would be applicable to a wide range of species with acceptable specificity. We therefore attempted to design a universal PCR outside this region. Considering the PCR efficiency calculated and the product length predicted, the primers for the universal PCR were set in the conserved regions shown as #7 and #10 in Table S3. These two nucleotide sequences each code a part of the stem in the secondary structure of the mammalian 16S rRNA molecule as advocated by Burk et al. [23]. The primer sequences matched another ten mammalian species (e.g., African elephant, sloth, mouse, capybara, rabbit, flying fox, horse, platypus, American opossum, and swamp wallaby), except one mismatch site [23]. The nucleotide sequence diversities of this PCR target among five species are summarized in Table 1 and ranged from 15.7% (pig vs dog) to 24.1%

Table 1 Nucleotide sequence diversities in the partial region of 16S rRNA (below the diagonal) and Cyt *b* (above the diagonal)[a]

	Human	Cow	Pig	Dog	Cat
Human		27.1%	24.9%	24.6%	24.1%
Cow	98/406 (24.1%)		17.9%	22.6%	19.7%
Pig	93/409 (22.7%)	66/409 (16.1%)		20.7%	19.7%
Dog	97/413 (23.5%)	67/411 (16.3%)	65/413 (15.7%)		21.1%
Cat	97/407 (23.8%)	64/406 (15.8%)	65/409 (15.9%)	65/410 (15.9%)	

[a] Results of the Cyt *b* are of the study published by Hsieh et al. [2]

(human vs cow), representing 65 nucleotide differences out of 413 compared (including gaps) to 98 out of 406. We can compare these values with the study reported by Hsieh et al. [2], which focused on a 402-bp-long partial region of Cyt *b* as the target of species identification (Table 1, above the diagonal). Although the nucleotide sequence diversity of our target is slightly lower than theirs, it is possible to state that it would still be a powerful tool for discriminating between species.

Consistency of nucleotide sequences within species

To verify the sequence consistency within each species, nucleotide sequences of the products in the universal PCR were analyzed in multiple individuals of each of the five species. These sequence data are currently in submission to GenBank. For convenience, nucleotide position (np) was numbered by calling a site next to 3′-terminal of the forward primer as number 1 in all species. A certain number of polymorphic sites were found in every species (Table 2), and four of the species, humans, cows, pigs, and dogs, showed less than five polymorphic sites. Most of them were observed in low frequency except for one site, np 290 in dogs, which showed 22 variants in a total of 53 individuals (42%). Lee et al. [24] reported nucleotide polymorphisms in the Cyt *b* region in 98 human individuals. They determined 1,118-base-long nucleotide sequences of the Cyt *b* coding region and found a total of 30 polymorphic sites, corresponding to 2.7%. Such a value in our target remained at 1.2% (in humans, five sites in 403-base-long). This supports that our target is highly conserved within species. In cats, a heteroplasmy was frequently observed during sequencing analyses. Figure S1 shows the electropherograms of both forward and reverse sequencing in a cat sample. The sequence quality dropped off dramatically beyond np 246 in forward and np 257 in reverse sequencing. This is probably due to a length heteroplasmy as observed in the human mtDNA control region with a high frequency in a similar image of the electropherogram [25, 26]. Of the 50 cat samples analyzed, 42 showed this length heteroplasmy. Additional internal sequencing was performed with inner primers listed in Table S2. However, there still remain the nucleotides which were impossible to determine from both the forward and reverse directions. Therefore, the nucleotides located between np 241 and 260 were left as unknown and omitted from comparisons for all cat samples. Another kind of heteroplasmy, heteroplasmic point mutation, was also observed in cat samples. This heteroplasmy was identified as clearly separated but mixed peaks in the electropherogram. Some examples are shown in Fig. 1. We called sites heteroplasmic only when a mixture of two peaks was observed both in forward and reverse sequencing. Of the

Table 2 List of the intraspecies polymorphic sites in five species

Species (*n* in total)	Position	Nucleotide	*n*
Human (50)	111[a]	T	45
		C	5
	257	T	45
		C	5
	278	C	49
		T	1
	316	G	48
		A	2
	320	C	49
		T	1
Cow (50)	8	T	49
		C	1
	248	G	42
		A	8
	275	T	42
		C	8
	279	C	48
		T	2
Pig (50)	166	A	41
		G	9
	234	T	44
		C	6
	251	C	41
		T	9
	368	C	49
		T	1
	371	T	49
		C	1
Dog (53)	243	T	41
		C	12
	248	A	52
		G	1
	290	A	31
		G	22
Cat (50)	155	T	25
		K[b]	20
		G	5
	282	A	26
		R[b]	18
		G	6
	319	T	20
		Y[b]	10
		C	20
	353	A	25
		M[b]	18
		C	7
	367	C	17
		Y[b]	17
		T	16
	368	T	41
		C	9
	378	A	24
		R[b]	19
		G	7

[a] Nucleotide numbering started from the first nucleotide next to the 3′-terminal of forward primer in all species.
[b] Heteroplasmic positions were called by IUPAC nucleotide code.

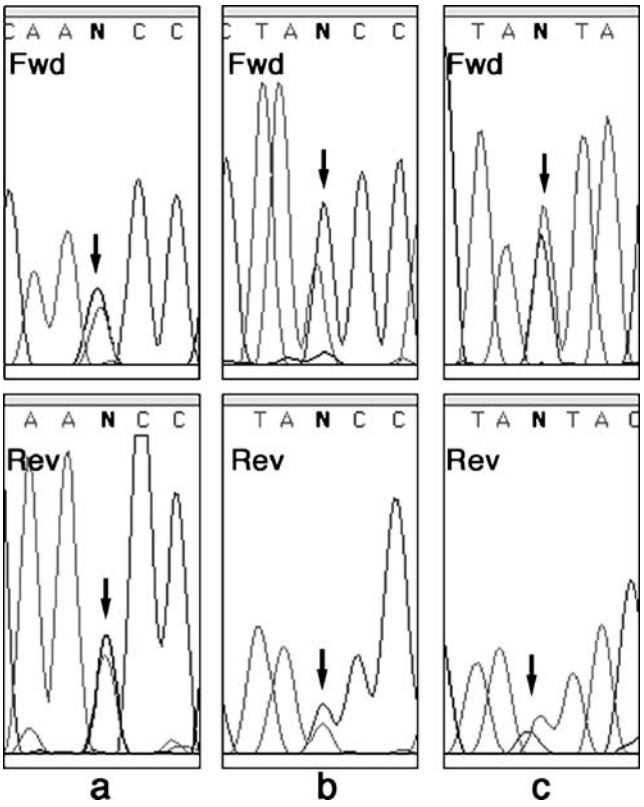

Fig. 1 Examples of heteroplasmic point mutation observed in the cat (F002) sample. Mixed peaks (*arrows*) are clearly observed in both the forward (*Fwd*) and reverse (*Rev*) sequencing at np 155 (*panel* **a**), np 353 (*panel* **b**), and np 367 (*panel* **c**)

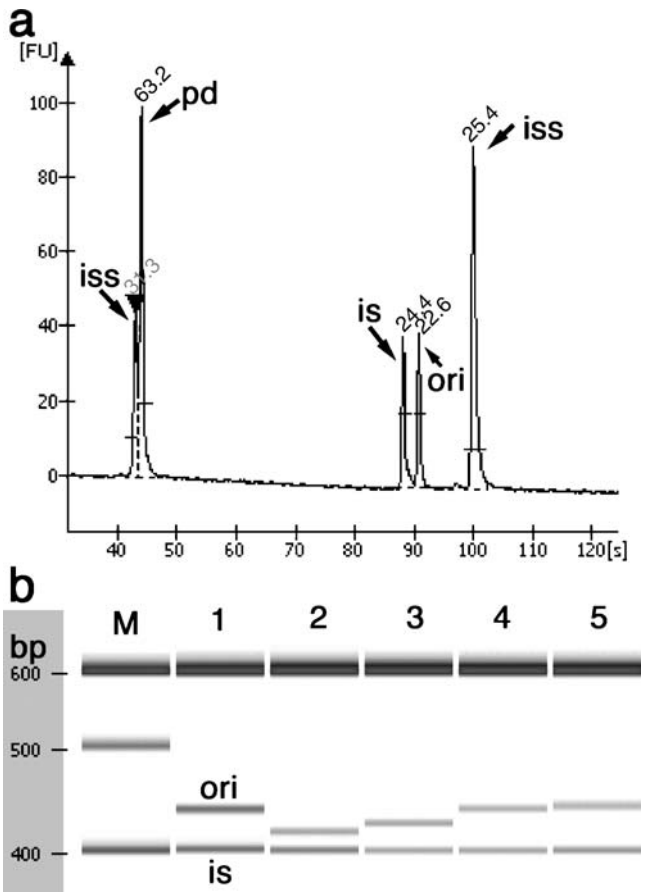

Fig. 2 Results of the competitive PCR. **a** The electropherograms in the competitive PCR of diluted human DNA with 1,000 copies of internal standard in universal PCR. The peaks that originated from sample-origin DNA (*ori*) and internal standard (*is*) are equivalent in the area (angled values). Other peaks originated from primer dimer (*pd*) and internal size standard (*iss*). **b** A gel-like image of which lanes were reconstructed from the results of electropherograms. *Lane M* 100 bp ladder marker, *lane 1* human, *lane 2* cow, *lane 3* pig, *lane 4* dog, *lane 5* cat. The bands indicated as "ori" and "is" correspond to the peaks in *panel* **a**

seven polymorphic sites found in cat samples, six showed a heteroplasmic mixture with high frequencies ranging from 20% (np 319) to 40% (np 155). All these sites, except np 155, were located outside of the putative stems of the mammalian 16S rRNA molecule model [23], so that most of the heteroplasmic point mutations observed in cat samples are thought to have little effect on the secondary structures of the molecule.

Competitive PCR

To assess the sensitivity of the PCR designed in this study, it was necessary to prepare the template DNA containing similar amounts of mtDNA template in each species. This preparation would be difficult because it requires strict separation of the mtDNA molecules from the extract containing huge amounts of nuclear DNA; otherwise, the quantity of DNA could not be as reliable as mtDNA. Consequently, we prepared DNA extracts containing similar amounts of amplifiable mtDNA using the competitive PCR method. Figure 2 shows the results of the competitive PCR in human DNA samples. Two peaks resulting from the competitive PCR are equivalent in area (Fig. 2a), indicating that the amplifiable DNA in the template corresponds to the amount of 1,000 copies. Such preparation was also performed for the other species, and the results of the competitive PCR are shown together as a gel-like image (Fig. 2b). The internal standard designed for this competitive PCR was homologous with regard to the sequences of priming sites. Bouaboula et al. [27] and Cottrez et al. [28] designed the competitive PCR with such a homologous competitor in different lengths of targets and demonstrated that the PCR efficiencies of the internal standard and of the target were consistent at every phase of the PCR regardless of the target length. These advantages of using the homologous competitor support the reliability of the competitive PCR developed in this study.

Validation of the PCR designed in this study

In this study, a total of six PCRs were designed, and the specificity and sensitivity were tested using DNA templates containing constant amounts of amplifiable mtDNA (1,000 copies). The results are shown in Fig. S2. It was found that every species-specific PCR correctly identified each target species without any cross-reaction with other species. Although the additional band due to a primer–dimer was observed in human-specific PCR in comparatively high numbers, these can easily be discriminated by size. The sensitivity of each PCR was slightly different from each other. Of the five species-specific PCRs, the one for the dog showed the lowest sensitivity even after lowering the annealing temperature to 54°C. Such unequal sensitivities among PCRs cannot easily be improved because the available regions for setting selective primers were limited in distribution. However, it could become acceptable to use these PCR systems for casework if we know each level of sensitivity. Preparation of the DNA template using the competitive PCR must be a correct approach for this assessment. Applicability of the PCR to degraded DNA was also tested in this study. The DNA extracted from the experimentally degraded bone specimens was submitted to each PCR (Fig. S3), and no influences on the accuracy of the PCR were observed.

Universal PCR vs species-specific PCR

The species-specific PCRs for five species were designed in this study. This type of testing method permits us to identify particular species directly by observing the achievements of the PCR, so that it contributes to shortening the time for the test. Such approach would be

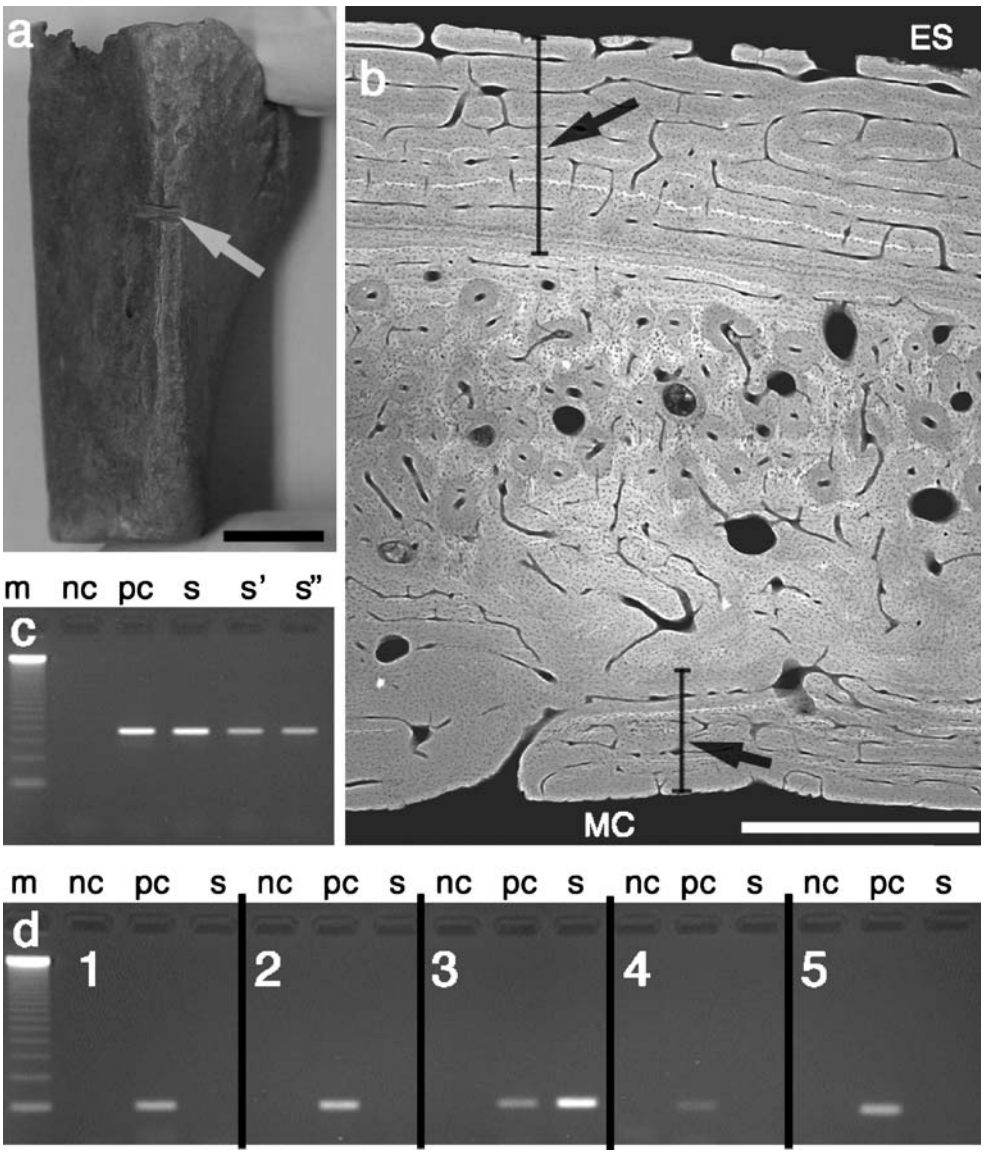

Fig. 3 A casework example for species identification. **a** A questioned bone particle. *Arrow* indicates a tool mark observed. *Bar* 1 cm. **b** Histological images of the specimen by X-ray microradiography. The areas showing lamellar structures are indicated by *arrows*. *Bar* 1 mm, *ES* external surface of bone, *MC* medullary cavity. **c** The result of universal PCR. *Lane m* 123 bp ladder marker, *lane nc* negative control, *lane pc* positive control (100 pg of human genomic DNA), *lanes s, s', s"* DNA from the specimen (*s* undiluted, *s'* 1:10 diluted, *s"* 1:100 diluted). **d** The results of species-specific PCR. *Lane m* 123 bp ladder marker, *lane nc* negative control, *lane pc* positive control (standard DNA of each species), *lane s* DNA from the specimen (undiluted), *set 1* human-specific PCR, *set 2* cow-specific PCR, *set 3* pig-specific PCR, *set 4* dog-specific PCR, *set 5* cat-specific PCR

particularly useful in the caseworks of human specimen to demonstrate that it is of human before processing the DNA profiling. Furthermore, species-specific PCR would play important role in cases where the sample is supposed to be mixed with more than two species of DNA, such as in a dog bite case [29]. The dog-specific PCR would be a good tool to show the existence of canine DNA even though the specimens (e.g., swab of the wound) must contain significant amount of human DNA originating from the victim. This preliminary test must increase the scientific certainty in following individualization by canine STR typing [7, 29, 30]. However, the species-specific PCR suffers disadvantages with regard to a multiplicity of the species to be tested. It is impossible to design specific primer sets for all species that should be covered in forensic field. In addition, this method cannot discriminate between an inhibitory effect and a lack of the target DNA when the PCR failed to be achieved. The method of the universal PCR and following sequencing can circumvent these problems. We should bear in mind these advantages and disadvantages in each approach for developing the forensic species identification test.

Case report

A part of a bone shaft was found in a sewage pipe (Fig. 3a). A side of the specimen appeared to have been cut transversely by a tool that was thought to be a high-speed cutter from its appearance. A dark-brown coloration reached to a deep layer of the compact bone, and the edge of the cross-section had lost its sharpness, suggesting a long-term immersion in raw sewage. A histological cross-section obtained by the X-ray microradiography method [31] strongly suggested that it was not of human origin due to a lamellar structure observed in the wide area of the cross-section [32–35] (Fig. 3b). To identify the species, DNA was extracted and analyzed using the PCRs developed in this study. A sufficient amount of the product was amplified in 1:100 diluted extract in the universal PCR (Fig. 3c). The sequencing result of this product revealed that the nucleotide sequence was consistent with the pig sequence in the GenBank database. The amplification was achieved only with the pig-specific PCR among the five sets of species-specific PCR (Fig. 3d). Considering these results, the gross morphology of the specimen was compared with of an adult pig skeletal preparation, and it was concluded that this specimen was part of the right tibia of a pig.

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